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## REVIEW ARTICLE

# Exploring the diversity of protein modifications: special bacterial phosphorylation systems

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**One sentence summary:** Investigating more unusual and less well-characterized bacterial tyrosine, arginine or serine/threonine kinase and phosphatase systems is not only important to understand their role in bacterial physiology but will help to generally understand the full potential and evolution of protein phosphorylation for signal transduction, protein modification and homeostasis in all cellular life.

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## ABSTRACT

Protein modifications not only affect protein homeostasis but can also establish new cellular protein functions and are important components of complex cellular signal sensing and transduction networks. Among these post-translational modifications, protein phosphorylation represents the one that has been most thoroughly investigated. Unlike in eukarya, a large diversity of enzyme families has been shown to phosphorylate and dephosphorylate proteins on various amino acids with different chemical properties in bacteria. In this review, after a brief overview of the known bacterial phosphorylation systems, we focus on more recently discovered and less widely known kinases and phosphatases. Namely, we describe in detail tyrosine- and arginine-phosphorylation together with some examples of unusual serine-phosphorylation systems and discuss their potential role and function in bacterial physiology, and regulatory networks. Investigating these unusual bacterial kinase and phosphatases is not only important to understand their role in bacterial physiology but will help to generally understand the full potential and evolution of protein phosphorylation for signal transduction, protein modification and homeostasis in all cellular life.

**Keywords:** protein kinase/phosphatase; protein-tyrosine phosphorylation; protein arginine phosphorylation; protein serine/threonine phosphorylation; protein homeostasis; protein modification

## INTRODUCTION: POST-TRANSLATIONAL MODIFICATIONS IN PROTEIN HOMEOSTASIS, FUNCTION AND REGULATION

Ribosomal protein synthesis and folding evolved using a limited set of proteinogenic amino acids, which are chemically diverse and reactive enough to cover many of the tasks proteins fulfill

in their specific cellular environment. However, to confer the diversity of protein functions, nature has also evolved a myriad of post-translational modifications (PTMs) of proteins in which some amino acids are covalently modified. These modifications can influence protein folding, activity and stability. In addition, they can control interactions with other macromolecules or cellular localization.

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PTMs are very diverse ranging from methylation, glycosylation, lipidation, carboxylation, nitrosylation, phosphorylation, acetylation and oxidation (Cain, Solis and Cordwell 2014). These various PTMs are not genetically encoded and can have many different outcomes for the affected protein and the cellular processes it is involved in (Prabakaran et al. 2012). In some cases, a specific modification of amino acids in a protein can alter, expand or enable their enzymatic activity. They can also specifically influence protein conformation or surface charge of a protein. Protein modifications, such as phosphorylation, methylation or acetylation, can thereby directly or indirectly change or enable interaction with proteins or other cellular components. Specific hydrophobic modifications such as lipidation can target the modified proteins to membranes (Walsh, Garneau-Tsodikova and Gatto 2005). PTMs such as glycosylation or addition of bacillithiol can also protect proteins and prolong their activity (Gaballa et al. 2010; Braakman and Hebert 2013). However, irreversible and uncontrolled protein modification mediated by for example oxidation, nitrosylation or generation of free radicals can damage proteins and facilitate misfolding with subsequent inactivation and aggregation. This is especially critical for proteins, which are already prone to unfolding due to environmental changes, such as temperature shifts or other stresses.

It is now clearly established that the vast majority of PTMs are ubiquitous among the three primary kingdoms of life, namely eukarya, archaea and bacteria, even if they were long neglected for the latter two (Walsh, Garneau-Tsodikova and Gatto 2005; Minguéz et al. 2012; Cain, Solis and Cordwell 2014). Among PTMs, protein phosphorylation, acylation and glycosylation are present in proteomes of bacteria, archaea and eukaryotes, supporting the ancient root of these modifications (Hu, Lima and Wolfe 2010; Wolfe 2010; Beltrao et al. 2013; Sousa et al. 2013; Hentchel and Escalante-Semerena 2015). By contrast, PTMs involving small proteins such as Ubiquitin, the related SUMO of eukaryotic cells (Ciechanover 2005) and Pup from mycobacteria or SAMP in archaea (Maupin-Furlow 2014), were identified to act as covalently attached protein modifications. This suggests that such small protein modifiers could be added during evolution to the already existing more ancient cellular repertoire of protein modifications, possibly after the development of the different cell types of the three domains of life.

It would be reasonable to assume that during evolution the presence of protein modifications and their potential influence on protein function and activity coevolved. Concomitantly, the control of protein modifications by modifying and de-modifying enzymes must be an intrinsic aspect of protein homeostasis and folding. Their activities can, for example, protect proteins from detrimental or excessive modifications, which could influence or impair their folding, function and activity.

Furthermore, it is well established that protein modifications play an important direct role in controlling cellular protein homeostasis and quality control. One well-studied example in eukaryotic cells is the protein quality control system of the endoplasmic reticulum. Here, unfolded and folded proteins are monitored and specifically labeled by different glycosylation modifications, mediating chaperone recognition (Braakman and Hebert 2013; Hebert et al. 2014). Another prominent example is the small protein ubiquitin, which was first discovered as a removable specific tag that targets proteins for degradation by the proteasome (Ciechanover 1998, 2005).

However, many of the proteins, enzymes and activities involved in the different reversible and transferable post-translational modifications could also have a great potential to

become important components of more complex cellular signal sensing and transduction networks. Cells must be able to sense and respond to intra- and extracellular changes and conditions. This can include sensing nutritional states, environmental changes and cell-cell communication amongst many other processes. These signal transduction networks must directly or indirectly sense a signal, often through membrane embedded receptor proteins. They utilize proteins which add ('writer') or remove ('eraser') a post-translational modification and proteins or protein domains, which are able to sense that signal for example by recognizing and interacting with modified proteins ('reader'). Such elements enable cellular and subcellular signal transduction cascades and networks, which can lead to a PTM of a regulatory protein resulting in a transcriptional, translational or post-translational controlled cellular response (Lim and Pawson 2010; Beltrao et al. 2013).

In summary, during evolution specific new cellular functions and pathways can be established by reversible protein modification. Not only one, but several protein modifications can be reversibly added to a given region in a protein, and their effect on protein function can be additive or combinatorial. The addition of a modification can also synergistically influence the addition of other protein modifications (Beltrao et al. 2012, 2013; Minguéz et al. 2012; van Noort et al. 2012).

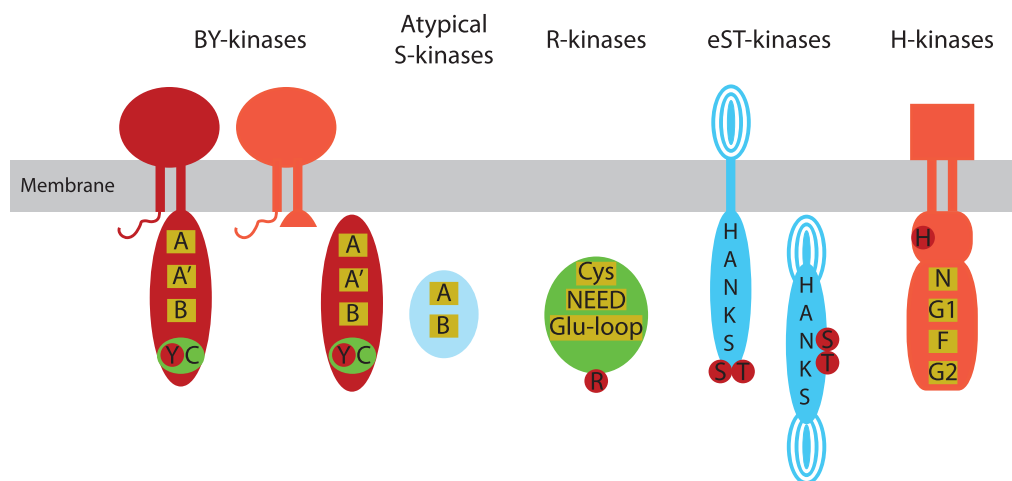
Among bacterial PTMs, phosphorylation is probably the most documented and the one for which we have gained the best understanding of its biological function. In contrast to eukaryotes, bacteria contain several families of enzymes that catalyze this modification and that are able to phosphorylate a wide range of amino acids (Fig. 1). Over the last 15 years, rather atypical enzymes, restricted to bacteria, have been shown to function in bacterial protein phosphorylation. In this review, after a brief overview of the well-known bacterial phosphorylation systems and the different types of amino-acids that are phosphorylated, we will focus our description on the recent findings concerning atypical tyrosine-, serine- and arginine-kinases and discuss their potential in bacterial physiology (Fig. 1).

## PHOSPHORYLATION AS POST-TRANSLATIONAL PROTEIN MODIFICATION IN BACTERIAL SIGNAL TRANSDUCTION NETWORKS

### Implication of different chemistries for phosphate modifications

Phosphate is an essential and ancient component of many basic molecules which define cellular life (Westheimer 1987). Therefore it comes as no surprise that phosphorylation was the first covalent protein modification influencing protein function discovered and characterized in the 1950's (Cohen 2002). Already in 1943, Cori and Green hypothesized that the enzyme, which converts glycogen phosphorylase from type 'a' to type 'b', catalyzes the removal of tightly bound adenylate (Cori and Green 1943). More than a decade later, Fischer and Krebs established that this conversion depends on reversible enzymatic attachment and removal of phosphate (Fischer and Krebs 1955). However, it should be mentioned that the enzymatic phosphorylation of a model protein substrate was described a little earlier by Burnett and Kennedy (Burnett and Kennedy 1954).

As it turned out, protein phosphorylation is an essential part of many signal transduction pathways, where a signal is sensed and transmitted via a network, which can contain protein



**Figure 1.** 'The five types of bacterial protein-kinases.' From left to right: bacterial tyrosine-kinases (BY-kinases) are either found in the form of a single-membrane protein (in red) or two separate polypeptides (in orange and red). Interaction between the two polypeptides is required for kinase activation. The cytoplasmic part constitutes the catalytic site, which possess three Walker motifs (A, A' and B) (yellow boxes) that are required for ATP binding and hydrolysis. They are able to autophosphorylate on several tyrosine residues (red circle) in a C-terminal motif termed tyrosine cluster (YC, green circle). The same is true for atypical serine-kinases (S-kinase) except they do not possess a Walker A' motif and are unable to autophosphorylate. Arginine-kinases (R-kinases) possess a conserved Cysteine (Cys), a NEED segment and glutamate located in a flexible loop (Glu-loop). These three domains stabilize and orient the substrate arginine in the transition state of the phosphate transfer reaction. Eukaryotic-like serine/threonine-kinases (eSTK) are either cytoplasmic or inserted in the membrane. Their kinase domain contains the 12 conserved signatures defined by Hanks. Both of them possess a variety of additional subdomains (illustrated by the blue and white striped ovals) that regulate their activity or that are proposed to influence their cellular localization and/or to favor their interaction with their substrates. They are able to be autophosphorylated on, but not exclusively, their activation loop. Membrane histidine-kinases (H-kinases) from two-component systems are composed of an extracellular sensory domain linked to a cytoplasmic domain required for catalysis and dimerization. The latter possess a conserved histidine residue (red circle) undergoing autophosphorylation. The catalytic domain contains four conserved characteristic sequences (N, G1, F, G2 and yellow boxes) required for ATP binding. Atypical S-kinases and H-kinases are specifically involved in the control of metabolism and the regulation of gene expression, respectively. By contrast, BY-kinases and eSTKs phosphorylate a large array of proteins and regulates several aspects of the bacterial physiology. All types of protein-kinases are widespread in bacteria. However, others like the atypical S-kinase IDHK/P are restricted to some species.

kinases, phosphatases and phosphoprotein sensing proteins (Cohen 2002; Hunter 2014; Kyriakis 2014). Phosphates can be transferred in these signal-transduction networks via a cascade of signal transduction proteins, allowing the integration of different regulatory networks (Hunter 2000, 2014; Mitrophanov and Groisman 2008; Harrison 2012; Morrison 2012).

The proteinogenic amino acids, which can become phosphorylated in bacteria, form different chemical bonds. Serine, threonine and tyrosine form a simple ester bond when phosphorylated. Histidine, arginine or lysine, form a phosphoramidate P–N bond, and aspartate a mixed anhydride or acyl phosphate, while phosphorylated cysteine forms a phosphorothiolate P–S bond. The different thermodynamic and kinetic properties of the chemical bonds between the phosphate group and the respective amino acid can result in altered kinetic stability and transfer potential of these phosphate groups. Furthermore, these different chemical properties can lead for example to altered biological function in signal transduction networks. (Teague and Dobson 1999; Stock, Robinson and Goudreau 2000; Cohen 2002; Fuhrmann et al. 2009; Elsholz et al. 2012; Hunter 2012; Kee and Muir 2012; Sun et al. 2012; Bertran-Vicente et al. 2014; Buchowiecka 2014; Fuhrmann, Clancy and Thompson 2015).

The phosphoester bond in phosphorylated serine, threonine or tyrosine is thermodynamically quite stable compared to phosphoramidate or acyl-phosphate bonds (Teague and Dobson 1999; Stock, Robinson and Goudreau 2000; Fuhrmann et al. 2009; Hunter 2014). Therefore, the presence of specific, possibly also regulated, phosphatases to reverse these modifications might be more warranted, especially when they are part of signal transduction networks (Dworkin 2015). One chemical distinction between serine- or threonine-phosphate and tyrosine-phosphate

is the comparably long distance of the phosphoester group to the peptide chain, due to the *para* position of the hydroxyl in the benzene group of tyrosine. This more exposed position of the phosphate might facilitate a better interaction with phosphotyrosine-binding protein domains (Hunter 2014).

By contrast, the phosphoramidate bond in phosphorylated histidine and the acyl phosphate of phosphorylated aspartate are relatively high-energy bonds. Interestingly, it was even suggested that the free energy of acyl phosphate bonds of proteins phosphorylated at specific aspartates might support conformational changes, important for signal transduction. The different chemical properties of the phosphate bond in the bacterial two-component system, compared to e.g. eukaryotic serine/threonine or tyrosine kinase networks, results in a different flux and kinetic of phosphate transfer (Stock, Robinson and Goudreau 2000).

Phosphorylated arginine forms a high-energy phosphoramidate bond, whose free energy is even sufficient to fuel the conversion of ADP to ATP (Teague and Dobson 1999). Arginine-phosphate is used by eukaryotic kinases to buffer the ATP/ADP ratio in specific cells (Ellington 2001). The chemical properties of this high-energy bond suggests the possibility of the occurrence of phosphate transfer to other proteinogenic amino acids such as e.g. tyrosine or serine (Elsholz et al. 2012; Schmidt, Ammerer and Mechtler 2013). Therefore, it was a rather unexpected discovery that in Gram-positive bacteria modification by phosphorylation of arginines can occur in the context of proteins (Fuhrmann et al. 2009). It was demonstrated that specific protein arginine modifications can also have a regulatory function, because the positively charged guanidium side chain of arginine in a protein can, for example, functionally interact with negatively charged groups found in proteins, DNA or RNA.

Phosphorylation of the guanidium side chain practically reverses the positive to a negative charge, which can abolish these possible functional interactions (Fuhrmann et al. 2009).

The thiol-group in cysteine is the target of many oxidative modifications, but can also become phosphorylated. The difficulty to detect this protein modification might be related to the relatively high free energy of a phosphothioester bond (Sun et al. 2012). In protein-tyrosine phosphatases, an intermediate covalently phosphorylated cysteine is part of the enzyme mechanism (Fuhrmann et al. 2013; Buchowiecka 2014). Cysteine-phosphorylation was also detected as an intermediate in the bacterial phosphoenolpyruvate-phosphotransferase system (PTS) (Mijakovic and Macek 2012; Deutscher et al. 2014). Recently, regulatory cysteine phosphorylation was also shown to be involved in the control of transcription factors in a regulatory network of the Gram-positive bacterium *S. aureus* (Sun et al. 2012).

Last, very little is known about a possible biological role of phospho-lysine modifications, which could be detected *in vivo*; however, no lysine phosphorylating kinase has been identified yet (Bertran-Vicente et al. 2014).

### Kinase and phosphatase systems in bacteria

Bacteria utilize a wide variety of protein modification systems. The first identified and best characterized phosphorylation systems in bacteria are two-component systems (for review see Hoch 2000; Klumpp and Krieglstein 2002; Gao and Stock 2009; Goulian 2010; Capra and Laub 2012; Francez-Charlot et al. 2015) (Fig. 1). These systems are widespread in all bacterial species and consist of signal sensing histidine kinases, which are often membrane localized and use ATP to autophosphorylate themselves on a histidine residue. Upon signal-induced autophosphorylation, these kinases can transfer the phosphate on an aspartate of the receiver domain of a response regulator, which can use additional domains, such as DNA-binding or other effector domains, to translate the sensed signal to an appropriate cellular response. The histidine kinase itself can also act as a phosphatase for the response regulator, and in some regulatory networks additional phosphatases of the acylphosphate of receiver domains (Rap phosphatases) have a regulatory role. Two-component system proteins together with phosphotransfer proteins can also form complex and extended phosphorelay systems. The phospho-histidine and phospho-aspartate are relatively high-energy bonds, which can result in limited and short half-life times of the histidine and aspartate linked phosphates, compared to e.g. serine-phosphorylation. For some systems, this can be in the range of e.g. only two to about 30–60 min. Both protein modifications are difficult to directly detect by e.g. mass-spectrometry (Stock, Robinson and Goudreau 2000; Laub and Goulian 2007; Mitrophanov and Groisman 2008; Mijakovic and Macek 2012). Interestingly, variants of functioning response regulators, which lost their modifiable aspartate residue, were recently identified and characterized. Whether the modification of other residues or different signal transduction mechanisms control the activity of this new class of response regulators is actively investigated (Maule et al. 2015).

Protein histidine phosphorylation is also important for the bacterial PTS system, which is involved in sensing and regulating bacterial carbohydrate metabolism. There are also examples of serine- and cysteine-phosphorylations, which were described in the context of the PTS system of different bacteria (Mijakovic and Macek 2012; Deutscher et al. 2014).

Bacteria contain many Hanks-type serine/threonine kinases also known as bacterial eukaryotic-like serine/threonine kinases (eSTKs) (Fig. 1), and related phosphatases. This phosphorylation system is turning out to be a hallmark of bacterial signaling and several recent reviews already describe in detail its multiple roles in bacterial central and secondary metabolism, cell cycle and virulence (Mijakovic and Macek 2012; Canova and Molle 2014; Wright and Ulijasz 2014; Dworkin 2015; Manuse et al. 2016). One should however note that a Hanks-type serine/threonine kinase and its phosphatase (Stk1/Stp1) can also control cysteine-phosphorylation (Sun et al. 2012).

Besides two-component systems, PTS and eSTKs systems, two other major systems catalyzing tyrosine (BY-kinases) or arginine (McsB) phosphorylation have emerged over the last 15 years (Grangeasse et al. 2003; Mijakovic et al. 2003; Fuhrmann et al. 2009) (Fig. 1). Moreover, some enzymes catalyzing either, both phosphorylation and dephosphorylation (IDHK/P and HPrK/P) or sharing no resemblance (PtkA) with enzymes of the systems described above, have been identified in some bacterial species. Altogether, this suggests that the number of enzymes dedicated to protein phosphorylation is likely underestimated in bacteria. In the three following chapters, we detailed the current knowledge about these enzymes.

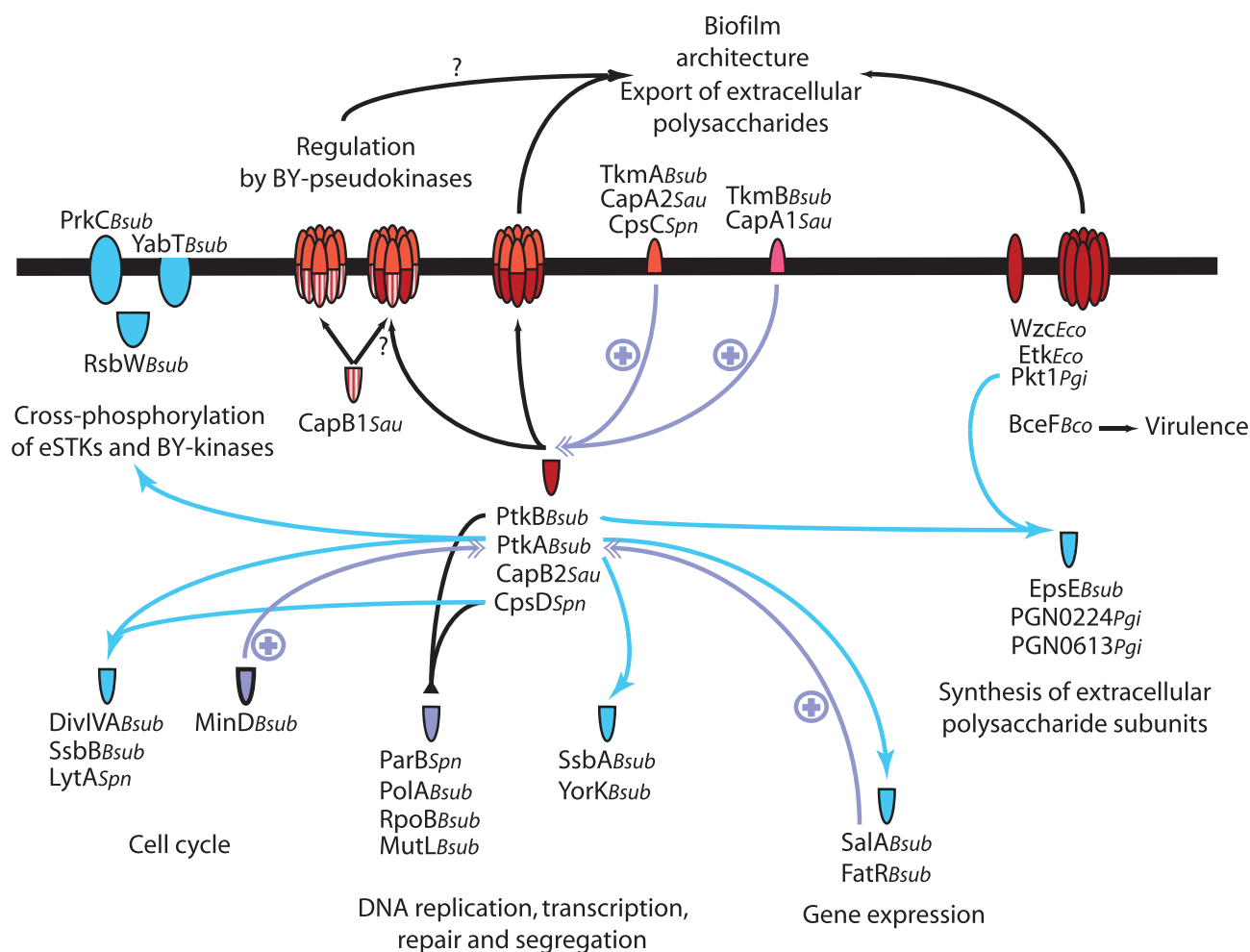
## PROTEIN-TYROSINE PHOSPHORYLATION IN BACTERIA

### Structure, molecular mechanism and substrate recognition of BY kinases

Genes encoding protein-tyrosine phosphorylation systems are widespread, but not omnipresent, in sequenced bacterial genomes (Shi et al. 2014a). A large majority of protein-tyrosine kinases identified and functionally characterized in bacteria belong to the family of bacterial protein-tyrosine kinases (BY-kinases) (Grangeasse et al. 2007) (Fig. 1). BY-kinases are defined by several structural and functional motifs (Grangeasse, Nessler and Mijakovic 2012). Their active site is situated in a catalytic domain capable of binding ATP and transferring its gamma phosphate to the hydroxyl group of tyrosine residues (Lee et al. 2008; Olivares-Illana et al. 2008). This domain is characterized by four conserved structural motifs. The Walker A and B motifs usually found in ATPases and GTPases constitute the ATP-binding site (Fig. 1). One should however note that BY-kinases also possess an additional Walker B motif located between the A and B motifs and termed A'. A much less conserved C-terminal sequence-motif with several clustered tyrosines represents the autophosphorylation site. The exact length of the tyrosine cluster and the position of autophosphorylated tyrosines can vary considerably among BY-kinases. This peptide is highly flexible to allow phosphorylation of all tyrosine residues. When dephosphorylated, BY-kinases form octamers in which the tyrosine cluster of one monomer locates in the catalytic site of the neighboring monomer. The intrinsic flexibility of the tyrosine cluster thus allows phosphorylation of all tyrosine residues. When phosphorylated, the octamer dissociates and forms monomeric BY-kinases allowing interaction with and phosphorylation of cellular substrates (Olivares-Illana et al. 2008).

Besides this general scheme, one should note that the importance of autophosphorylation for BY-kinase activity can vary from kinase to kinase. For instance, autophosphorylation of the *Bacillus subtilis* BY-kinase PtkB (EpsB) was reported to inhibit the kinase activity (Elsholz, Wacker and Losick 2014). Moreover, the kinase activity of the *B. subtilis* BY-kinase PtkA is not affected





**Figure 2.** 'Schematic model of Bacterial Tyrosine Kinases (BYK) functions'. BYKs are shown in red. They are activated (illustrated by double purple head-arrows and '+' signs) upon interaction with either their typical transmembrane modulators (orange and pink) or the cell cycle protein MinD and the transcriptional regulator SalA. Blue arrows and blue half-ovals show phosphorylation substrates of BYKs [substrates shown complete the list previously reported in (Bechet et al. 2009)]. Pseudo-BYKs are shown as red and white striped half-ovals. They can either form homo-octamers or presumably interact with BYKs to influence the biosynthesis and the export of extracellular polysaccharides. The black triangle shows proteins that interact with BYKs and for which phosphorylation has not been demonstrated. Bsub for *Bacillus subtilis*, Spn for *S. pneumoniae*, Sau for *S. aureus*, Eco for *E. coli*, Pgi for *P. gingivalis* and Bco for *B. contaminans*.

by autophosphorylation, and this kinase remains perfectly capable of substrate phosphorylation, even when all of its C-terminal tyrosines get replaced by phenylalanine (Mijakovic et al. 2003). Interestingly, some proteins possessing all the features of BY-kinases are unable to catalyze protein phosphorylation and are considered catalytically inactive *in vitro*. This is notably the case of CapB1 from *Staphylococcus aureus* whose atomic structure is similar to that of the active CapB2 homolog (Gruszczuk et al. 2013). On this basis, it is proposed that some BY-kinases could be inactive even if it cannot completely be ruled out that CapB1 could be active under specific *in vivo* circumstances.

This situation is nevertheless reminiscent of that observed in eukaryotes in which inactive pseudo-kinases act as molecular scaffolds or allosteric factors to promote the activity of other protein-kinases (Jura et al. 2009; Qin and Wu 2012). It is thus speculated that CapB1 could behave as a pseudo-kinase modulating the organization and the function of the other *S. aureus* BY-kinase CapB2 (Gruszczuk et al. 2013) (Fig. 2). Interestingly, the same was demonstrated for the eSTK PknB of *Mycobacterium tuberculosis* whose function in peptidoglycan biosynthesis is influenced by the pseudo-eSTK MviN (Gee et al. 2012).

The catalytic domain of BY-kinases requires an activator domain to reach its full catalytic potential. The activator domain enhances the ATP-binding affinity of the catalytic domain, and may assist in positioning the protein substrate (Grangeasse et al. 2003; Mijakovic et al. 2003). These activator domains are typically transmembrane, with the C-terminus pointing to the cytosol and interacting with the catalytic domain. The catalytic and activator domains of BY-kinases can exist separately (encoded by two different genes) or in a single polypeptide chain. If one examines the distribution of these two architectural prototypes in different bacterial phyla, one can conclude that the two-protein prototype is very common in firmicutes, whereas the single-protein prototype is most widely represented in proteobacteria (Jadeau et al. 2012; Shi et al. 2014a). In proteobacteria, an additional arginine- and lysine-rich domain, named RK cluster, contributes to ATP binding and/or hydrolysis (Bechet et al. 2010).

We have speculated previously that the separation of the catalytic and the activator domain in firmicutes may allow the catalytic domain to interact with alternative activators, and such has proven to be the case. In *B. subtilis*, it was recently shown

that BY-kinases PtkA and PtkB (EpsB) can 'swap' their activators, and PtkA can also interact with other proteins such as MinD, which stimulate its kinase activity (Shi et al. 2014c; Gao et al. 2015) (Fig. 2). The same is true for the two activators CapA1 and CapA2 that both activate the BY-kinase CapB2 of *S. aureus* (Gruszczyk et al. 2013). Interestingly, a recent study revealed that the C-terminus of the BY-kinase activator CpsC in *Streptococcus pneumoniae* flips across the membrane, and oscillates between the extracytosolic state (in 30% of cells) and cytosolic state (in 70% of cells), in which it can activate the BY-kinase CpsD (Whittall, Morona and Standish 2015). This could also indicate that in the cells in which the CpsC tail is pointing outwards, CpsD has to interact with an alternative activator protein.

The catalytic domain of BY-kinases, with the three Walker motifs, is in evolutionary terms most closely related to the ATP-binding domains of the arsenite ATPases (Rosen et al. 1999), the cell cycle regulators such as MinD (Ma, King and Rothfield 2004) and the ParA type protein Soj (Murray and Errington 2008). It has been suggested that these different protein families emerged through domain duplication and consecutive divergent evolution (Grangeasse, Nessler and Mijakovic 2012).

More recently, Chao and coworkers (2014) proposed that BY-kinases belong to a superfamily of bacterial protein-tyrosine kinases. Distantly related members of this superfamily would thus comprise Hanks-type protein-tyrosine kinases (Thomasson et al. 2002; Johnson and Mahony 2007; Arora et al. 2012), and a number of protein-kinases of diverse origins were classified by Chao, Wong and Av-Gay (2014) as 'odd'. This last category comprises PtkA from *Mycobacterium tuberculosis* (Bach, Wong and Av-Gay 2009), DivL from *Caulobacter crescentus* (Wu et al. 1999) and WaaP from *Pseudomonas aeruginosa* (Zhao and Lam 2002). We do not oppose this classification, but would like to point out those members of this superfamily, notably BY-kinases, share much more sequence homology with the ATPases mentioned above, than with the other kinase members of the superfamily proposed by Chao, Wong and Av-Gay (2014).

The recent finding that the BY-kinase CpsD could behave as a ParA-like protein strengthens this claim (Nourikyan et al. 2015). In addition, Shi et al. (2014a) recently reported a phylogenomics study, comparing all sequences of BY-kinases identified in bacterial genomes that were known at the time. BY-kinase was found in 577 out of 1471 bacterial genomes. Several interesting observations emerged from this evolutionary study. BY-kinase genes accumulated non-synonymous mutations about 3-fold faster than the genes used as control. By contrast, their rate of accumulating synonymous mutations was the same as for the control genes. The authors argued that the consequence of this increased mutability of BY-kinase genes is the lack of their coevolution with substrate proteins (phosphorylated by the kinases). This in turn led to the ability of BY-kinases to adopt new substrates in the course of evolution, and thus evolve new regulatory mechanisms as means of bacterial adaptation (Shi et al. 2014a). This hypothesis is supported by the evidence of 'promiscuity' of present-day BY-kinases, some of which have been shown to phosphorylate a number of different cellular proteins (Mijakovic and Deutscher 2015).

### BY kinases and extracellular polysaccharide synthesis

The ability of BY-kinases to phosphorylate cellular substrates and regulate their function, thus acting as signal transduction devices, has been extensively documented and reviewed (Grangeasse et al. 2007; Lee and Jia 2009; Shi et al. 2010; Grangeasse, Nessler and Mijakovic 2012; Whitmore and Lamont 2012).

Here, we will focus on some more recent findings that are presently shaping the field.

A large majority of BY-kinase genes are situated in operons dedicated to synthesis and export of extracellular (capsular- and exo-) polysaccharides. Early on, the autophosphorylation capacity of BY-kinases was correlated to the synthesis and export of these compounds, in which some BY-kinases and their activator proteins actively participate (Vincent et al. 2000; Wugeditsch et al. 2001; Bender, Cartee and Yother 2003). In *S. agalactiae*, it has recently been shown that the BY-kinase CpsD regulates the attachment of capsular polysaccharides to the bacterial surface by influencing the extracellular domain of its activator, CpsC (Toniolo et al. 2015). Recent evidence supports the possibility that BY-kinases also regulate extracellular polysaccharide production via protein substrate phosphorylation (Fig. 2). In *B. subtilis*, it could be demonstrated with both *in vivo* and *in vitro* experiments that the BY-kinase PtkB (EpsB) phosphorylates and activates the glycosyltransferase EpsE, involved in export of exopolysaccharide precursors (Elsholz, Wacker and Losick 2014). A comparable regulatory mechanism was suggested for the control of EpsE glycosyltransferase activity by the BY-kinase EpsD in *S. thermophilus* (Minic et al. 2007). PtkB (EpsB) activity is crucial for formation of structured biofilms in *B. subtilis* (Gerwig et al. 2014). In *Porphyromonas gingivalis*, the BY-kinase Ptk1 phosphorylates two enzymes involved in capsular polysaccharide production: an UDP-acetyl-mannosamine dehydrogenase and an UDP-glucose dehydrogenase. The mutant devoid of Ptk1 exhibits a loss of capsule, and by consequence loss of capacity to form a dual species community with *S. gordonii* (Wright et al. 2014) (Fig. 2).

### New regulatory functions of BY kinases

In addition to regulating extracellular polysaccharide production, recently interesting new functions of BY-kinases have been characterized (Fig. 2). Pneumococcal autolysin LytA, a major virulence factor, is phosphorylated by the BY-kinase CpsD, and this leads to premature autolysis (Standish, Whittall and Morona 2014). The BY-kinase BceF from *Burkholderia contaminans* has been shown to contribute to the epithelial disruption and invasion of cystic fibrosis lung epithelial cells (Ferreira et al. 2015).

Very recently, it was reported that the autophosphorylation of the BY-kinase CpsD acts as a signaling system coordinating capsular polysaccharide (CPS) synthesis with chromosome segregation to ensure that daughter cells are properly wrapped in CPS (Nourikyan et al. 2015). In this study, it was also shown that CpsD interacts with the chromosome partitioning protein ParB and that CpsD autophosphorylation modulates its mobility. However, ParB was not found to be phosphorylated by CpsD (C.G., unpublished observation). This suggests that BY-kinases could also regulate protein function by direct protein-protein interactions. This might also be a possible mechanism for PtkB (EpsB) of *B. subtilis* that was shown to interact with various proteins involved in DNA metabolism (Shi et al. 2014c). Bacterial protein-tyrosine kinases that do not belong to the BY-family also actively contribute to virulence. For example, the Hanks-type protein-tyrosine kinase CtkA from *Helicobacter pylori* was shown to induce pro-inflammatory cytokines and assist in bacterial persistence by escaping the host innate immune response (Tenguria et al. 2014). Some Hanks-serine/threonine kinases (eSTKs) of *Mycobacterium tuberculosis* were shown to function as dual-specificity kinases that also phosphorylate diverse proteins on tyrosine. It is proposed that this property would regulate some essential functions relating to growth (Kusebauch et al. 2014).

New functions of BY-kinases are also being discovered in non-pathogenic bacteria. In *B. subtilis*, the BY-kinase PtkA was shown to phosphorylate two transcription regulators, and control their physiological functions. PtkA-dependent phosphorylation of the transcription regulator FatR inhibits its binding to the target DNA sequence, and induces expression of FatR-regulated genes (Derouiche et al. 2013). By contrast, PtkA-dependent phosphorylation of the transcription regulator SalA enhances its binding to the target DNA, leading to repression of its target gene *scoC* (Derouiche et al. 2015) (Fig. 2).

### Phosphotyrosine-protein phosphatases

Protein-tyrosine kinases are usually described as the key signal-transduction players, and the corresponding phosphatases usually receive less attention, under the pretext that they play a more passive role and dephosphorylate phosphorylated proteins less selectively. Pathogenic bacteria possess phosphotyrosine-protein phosphatases whose main role is to be delivered to the host cell and scramble the signaling therein (DeVinney, Steele-Mortimer and Finlay 2000). Given their role, structurally and functionally they resemble the phosphatases from the host cells. The role of bacterial enzymes, which dephosphorylate phosphotyrosine-proteins is commonly accomplished by two types of phosphatases. In proteobacteria, the majority of phosphotyrosine-protein phosphatases belong to the family of low-molecular-weight phosphatases (LMW-PTPs) (Grangeasse et al. 1998). In firmicutes, LMW-PTPs are also common, but another family of phosphatases is present. These phosphatases resemble the histidinol phosphate phosphoesterases (Morona et al. 2002; Mijakovic et al. 2005). A number of recent studies highlight the specific roles of phosphotyrosine-protein phosphatases in controlling biological processes. In terms of controlling the synthesis of secreted polysaccharides, phosphatases are known to control the autophosphorylation state of kinases, which in turn controls their oligomerization state (Obadia et al. 2007; Standish and Morona 2014).

Phosphatases are often used as biochemical 'weapons' by bacteria that interact with eukaryotic hosts. For example, *P. syringae*, a plant pathogen, employs a secreted phosphotyrosine-protein phosphatase HopAO1 to dephosphorylate the pattern recognition receptor on the surface of plant cells. This leads to diminishing the immune response of the plant (Macho et al. 2014). *Mycobacterium tuberculosis* escapes the apoptotic activity of microphages by employing PtpA, a phosphotyrosine-protein phosphatase that dephosphorylates the host protein GSK3 $\alpha$ , and modulates its anti-apoptotic activity (Poirier, Bach and Av-Gay 2014). Interestingly, PtpA can be phosphorylated by a cognate kinase PtkA. PtpA specifically phosphorylated at the residues Y128 and Y129 exhibits an enhanced phosphatase activity, and is thus effectively regulated by the kinase (Zhou et al. 2015).

### Activation of BY-kinases

The current trend in the field of bacterial protein-tyrosine phosphorylation is an increased rate of discovery of physiological substrates of BY-kinases. Many of them are shown to phosphorylate multiple proteins, which is in accord with the evolutionary trend to maintain them as promiscuous regulatory systems (Shi et al. 2014a). However, there are very few studies that explain how the kinases get activated and their activity directed towards specific substrates. If they are to serve as signal transduction or regulation devices, they cannot constitutively phosphorylate all their substrates at the same time. One possibility is

that BY-kinases are activated by environmental cues, conveyed by specific ligands. An example is extracellular polysaccharide (eps) acting as a ligand, which regulates PtkB (EpsB) kinase activity by specific interaction with the extracellular domain of EpsA the modulator of PtkB (EpsB) (Elsholz, Wacker and Losick 2014). Another possibility is that the kinases interact with different protein activators at different times, and these activators direct their activity towards a specific subset of substrates, in putative kinase/activator/substrate complexes (Shi et al. 2014c). The activators themselves may not necessarily be dependent on environmental cues, but could have variable localization and propensity to interact with the kinase. More research will be needed to clarify this question, which is critical for full understating of the physiological role of bacterial protein-tyrosine phosphorylation.

## UNUSUAL SERINE/THREONINE PHOSPHORYLATION IN BACTERIA

### Unusual dual-function kinases/phosphatases related to BY-kinases

One of the distinguishing features of BY-kinases is that the Walker motifs, usually found in NTPases, constitute their active site. In that sense, they are quite unique in the world of protein kinases. In fact, there are only two other types of protein kinases, which are known to employ the Walker motifs as a kinase active site, and they are both found only in bacteria. These are the Hpr kinase/phosphorylase (HprK/P) (Deutscher and Saier 1983) and isocitrate dehydrogenase kinase/phosphorylase (IDHK/P) (Nimmo et al. 1984). A remarkable feature of these two kinases is that they are bifunctional enzymes; they are also capable of dephosphorylating their protein substrates. Structures of both enzymes have been resolved (Fieulaine et al. 2001, 2002; Singh et al. 2001; Zheng and Jia 2010). In both enzymes, the same active site catalyzes both the phosphorylation and the dephosphorylation reaction (Stueland, Ikeda and LaPorte 1989; Fieulaine et al. 2002; Mijakovic et al. 2002). However, dephosphorylation is not simply a reverse reaction of phosphorylation. In case of HprK/P, free phosphate is the acceptor of the exiting phosphate moiety during dephosphorylation, and the mechanism was thus termed phosphorolysis (Mijakovic et al. 2002). Since the two activities are distinct, it was possible to isolate mutants of residues in the catalytic sites abolishing one of the two activities (Ikeda, Houtz and LaPorte 1992; Monedero et al. 2001). Both HprK/P and IDHK/P act via substrate phosphorylation. HprK/P phosphorylates the histidine carrier protein HPr at the regulatory residue serine 46 (Deutscher and Saier 1983). Phosphorylated HPr interacts with a global gene regulator CcpA, which in firmicutes controls many operons involved in carbon catabolite regulation (Deutscher et al. 1995; Fujita et al. 1995). The signal which transmits the availability of glucose or other easily metabolized sugars to HprK/P is the glycolytic intermediate fructose-1,6-bisphosphate. This allosteric activator switches HprK/P into the kinase mode. This results in phosphorylation of HPr, interaction with CcpA and repression of operons involved in uptake of alternative carbon sources. When glucose gets depleted, HprK/P phosphatase activity takes over, and CcpA-dependent repression is alleviated, leading to expression of operons for utilization of alternative sugars. IDHK/P controls the phosphorylation state of the Krebs cycle enzyme isocitrate dehydrogenase. The metabolic state of the cell is transmitted to IDHK/P by the cellular levels of isocitrate, phosphoenolpyruvate, NADPH and the adenine nucleotides (Nimmo and Nimmo



1984). When growing on acetate, its kinase activity is stimulated and IDHK/P phosphorylates the isocitrate dehydrogenase. This inhibits the enzyme activity, and by consequence activates the glyoxylate shunt (Chung, Klumpp and LaPorte 1988). Dephosphorylation of isocitrate dehydrogenase reactivates the enzyme and shifts the metabolic flux back to the standard Krebs cycle. In conclusion, the dual activity of both HprK/P and IDHK/P is regulated by metabolic intermediates, and their role is to redirect the fluxes in the central carbon metabolism.

### The atypical serine/threonine kinase YihE

An intriguing example of an unusual protein-kinase was revealed by structural genomics. YihE is an *Escherichia coli* protein that shares little sequence similarities and no conserved signature with eSTKs. However, the structure of YihE shows an overall eSTKs-bilobal domain architecture with a N-terminal  $\beta$ -sheet domain and a C-terminal  $\alpha$ -helical domain (Zheng et al. 2007; Manuse et al. 2016). In addition, YihE was shown to autophosphorylate and to phosphorylate the myelin basic protein on serine and threonine only. *In vivo*, it is proposed that YihE would participate in expression of surface appendages (Zheng et al. 2007), and more recently, it was demonstrated that this kinase plays an important role in protecting *E. coli* cells against various lethal environmental stresses (Dorsey-Oresto et al. 2013).

To our knowledge, this case is unique to date, but it illustrates that other types of protein-kinases will emerge in the future from the reservoir of proteins with unknown function. In support of this, it should be noted that the genome of *E. coli* does not code for any eSTKs, whereas many proteins phosphorylated on serine and threonine have been detected *in vivo* by phosphoproteomics (Macek et al. 2008; Soung et al. 2009; Soares et al. 2013).

## PROTEIN ARGININE PHOSPHORYLATION, PROTEIN QUALITY CONTROL AND THE HEAT STRESS RESPONSE IN *B. SUBTILIS*

### Protein quality control networks and their regulation in *Bacillus*

Protein arginine phosphorylation in *B. subtilis* is intricately connected to various cellular stress response and protein quality control systems. The protein kinase McsB, which catalyzes protein arginine phosphorylation, is embedded in various levels of activities in the cellular protein quality control network of *B. subtilis* cells (Fig. 3). The McsB kinase activity is modulated by a number of different interacting proteins, whose activities can in turn be influenced by McsB (Fig. 4).

Protein quality control systems are essential to ensure cellular protein homeostasis and monitor the folding and active state of proteins in all cells. These highly conserved systems include chaperone systems but also AAA+ protease complexes such as Hsp100/Clp proteins in bacteria or the proteasome in eukaryotic cells. The chaperone systems can prevent protein aggregation [e.g. Hsp40 (DnaJ)], refold misfolded proteins [Hsp70/40 (DnaKJE); Hsp60 (GroE)] and even disaggregate and refold already aggregated proteins [Hsp100/70 (ClpB, DnaK)], while Hsp100/Clp and AAA+ proteases can remove misfolded and damaged protein species from the cellular environment by degradation (Fig. 3).

In *B. subtilis*, the genes and operons encoding chaperones and cochaperones of the Hsp70 (DnaK) and Hsp60 (GroEL) family are controlled by the repressor HrcA, which itself is activated by GroEL thereby monitoring the folding state of cellular pro-

teins (Mogk et al. 1997). Recently, it was demonstrated that Spx, a global regulator known to control the thiol stress response in *B. subtilis* (Nakano et al. 2003; Rochat et al. 2012), is also an important heat shock response regulator necessary for thermotolerance development (Runde et al. 2014), and its adaptor protein-mediated degradation can be controlled by intracellular protein aggregate accumulation (Engman and von Wachenfeldt 2015).

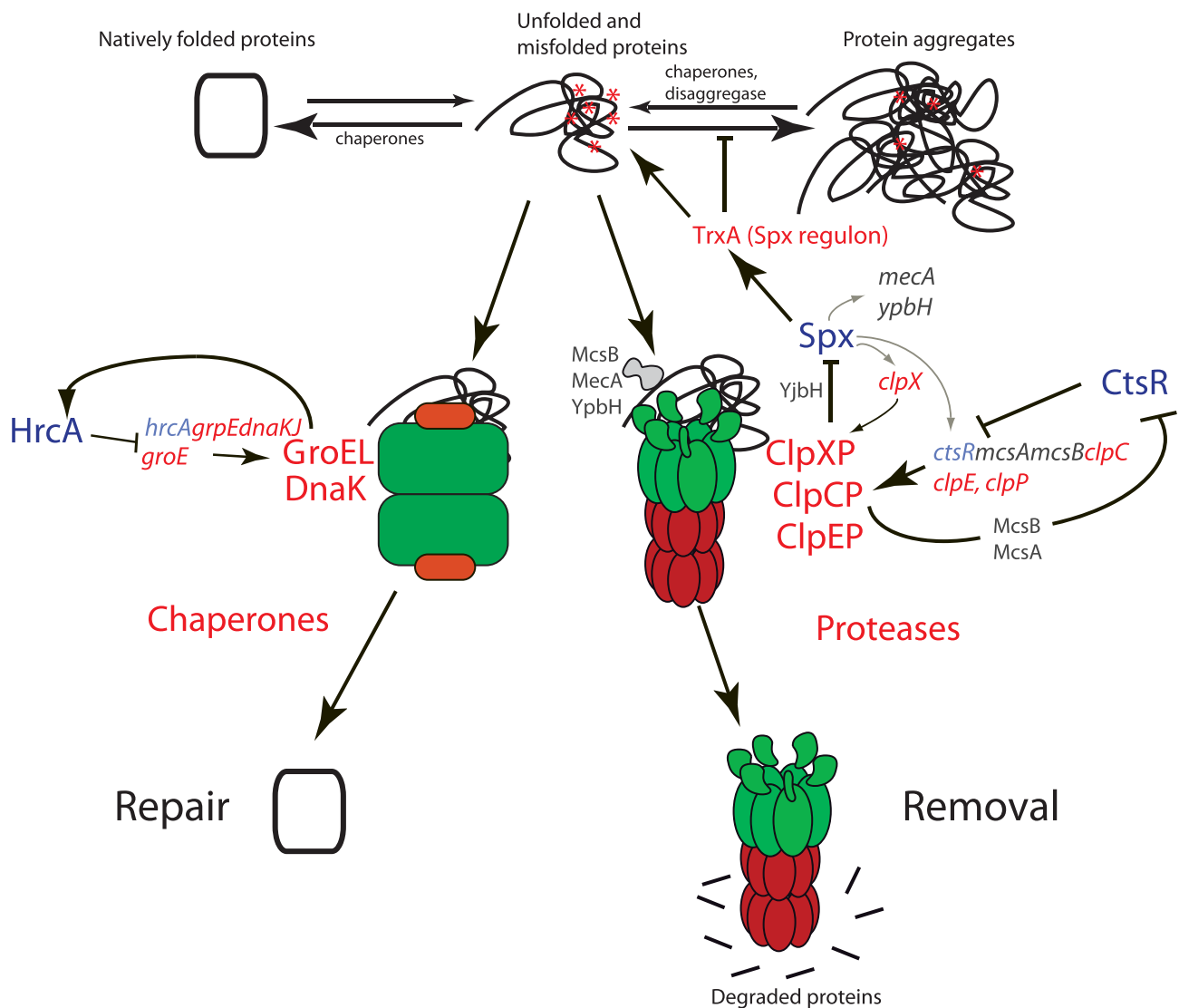
The other heat shock repressor of *B. subtilis*, CtsR, controls the transcription of the genes of ClpP and ClpE and the operon encoding CtsR, McsA, McsB and ClpC. CtsR itself can sense temperature, and its activity is also controlled by McsB (Krüger and Hecker 1998; Derré, Rapoport and Msadek 1999; Krüger et al. 2001; Fuhrmann et al. 2009; Elsholz et al. 2010) (Fig. 3).

Hsp100/Clp and AAA+ protease complexes are ATP driven molecular machines, which consist of two components (i) the hexameric Hsp100/Clp ATPase ClpC, ClpE or ClpX, which contains one or two core AAA+ domains with different additional loops and domains and (ii) the associated compartmentalized protease ClpP. Hsp100/Clp proteins can recognize and unfold proteins by ATP hydrolysis dependent translocation using specific loops in the pore formed by the AAA+ hexamer. The unfolded translocated proteins are subsequently transferred into the compartmentalized chamber, lined by proteolytic active sites, formed by the associated barrel-like oligomeric ClpP protease, where they are hydrolyzed into small peptides.

The ability to rescue and repair functional proteins by chaperones and/or to remove potentially toxic misfolded protein species by degradation could be considered as two branches of the same cellular protein quality control system (Fig. 3) (Wickner, Maurizi and Gottesman 1999; Tyedmers, Mogk and Bukau 2010; Hartl, Bracher and Hayer-Hartl 2011). Interestingly, the same AAA+ protease systems are concurrently involved in the signal transduction and control of cellular and developmental processes by regulatory proteolysis of e.g. transcription factors (Wickner, Maurizi and Gottesman 1999; Jenal and Hengge-Aronis 2003; Kirstein et al. 2009; Battesti and Gottesman 2013). ClpCP or ClpXP-mediated regulatory proteolysis controls, for example, a number of additional different regulatory networks in *B. subtilis* such as competence development or the thiol stress response (Turgay et al. 1998; Zuber 2004, 2009; Kirstein et al. 2009). Here, a regulatory protein such as a transcription factor like ComK or Spx is targeted by an adaptor protein Meca or YjbH for degradation by ClpCP or ClpXP. Upon a cellular signal the adaptor protein-mediated degradation is abolished and the regulatory protein immediately stabilized, resulting in a fast transcriptional reprogramming of the affected cells. An important aspect for regulatory proteolysis is that the adaptor protein activity can be itself regulated by specific signals (Turgay et al. 1998; Zhang and Zuber 2007; Kirstein et al. 2009; Battesti and Gottesman 2013; Engman and von Wachenfeldt 2015).

In general, adaptor proteins can expand and control the substrate recognition capabilities of their corresponding Hsp100/Clp proteins ClpC or ClpX. They can enable or enhance degradation by e.g. tethering substrates to the entrance pore of the AAA+ protein ring. Adaptor proteins like Meca or YjbH can interact with specific degrons in substrate proteins and concurrently bind to the respective N-terminal domain (NTD) of ClpC or ClpX (Kirstein et al. 2009; Sauer and Baker 2011).

The adaptor protein Meca of *B. subtilis* exhibits additional activities, because it interacts not only with the NTD but also the coiled-coil linker domain of ClpC and thereby supports the oligomerization and activation of ClpC, leading to the formation of the ClpCP complex (Kirstein et al. 2006; Wang et al. 2011). When not presenting substrate proteins to ClpCP, Meca itself is



**Figure 3.** 'Model of the *B. subtilis* heat shock regulation and protein quality control system'. Unfolded and misfolded cellular proteins can be repaired by chaperones (e.g. GroE and DnaK) or removed by Hsp100/Clp protease systems (ClpCP, ClpXP and ClpEP). The proteins and genes encoding chaperones and proteases are depicted in red, adaptor proteins, and their genes depicted in grey and their regulators (heat shock transcription factors HrcA, CtsR and Spx and their genes) are depicted in blue (Runde et al. 2014).

degraded by ClpCP, resulting in a deactivation of ClpC (Turgay et al. 1998).

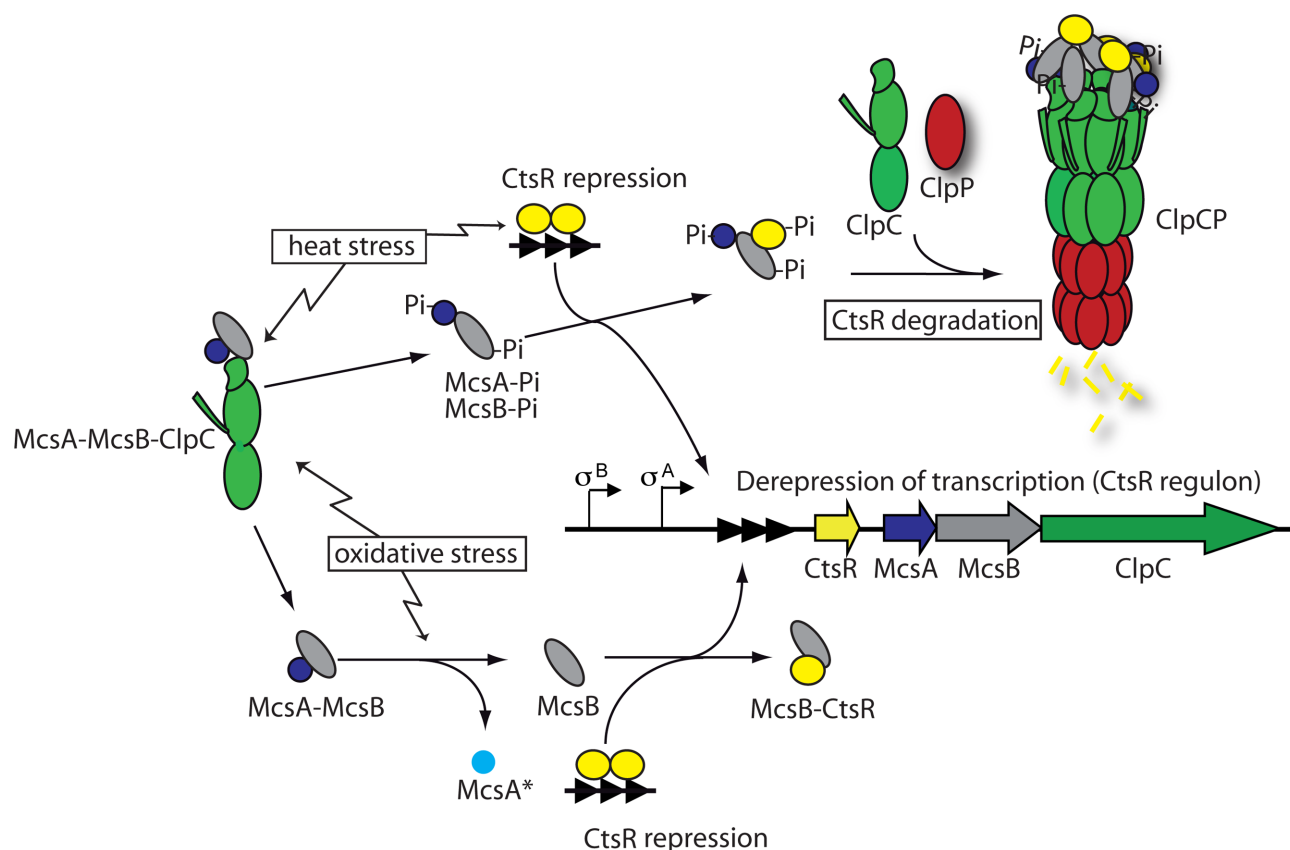
However, MecA can not only target a specific transcription factor for regulatory proteolysis to ClpCP but like its paralog YpbH can also facilitate the disaggregation of protein aggregates for refolding by chaperones in the absence or for general proteolysis in the presence of ClpP (Turgay et al. 1998; Schlothauer et al. 2003; Haslberger et al. 2008; Kirstein et al. 2009).

### McsB a protein kinase and adaptor protein

In order to discern the physiological role of cellular protein arginine phosphorylation and McsB, it is important to recognize (i) that McsB, McsA, ClpC and the heat shock repressor CtsR are intricately connected by protein–protein interactions, which enables them to mutually control their respective activities, (ii) that McsB in its active kinase form also functions as an adaptor protein, activating ClpC and concurrently targeting CtsR for ClpCP degradation and (iii) that the McsB protein arginine kinase ac-

tivity, which is modulated by McsA, ClpC and YwlE, is linked to most but not all McsB-mediated activities.

It was observed that McsB and McsA appear to directly modulate CtsR activity (Krüger et al. 2001). All three proteins can form a functional complex, where McsB itself directly inhibits CtsR activity, which is enhanced by McsA interacting with McsB. CtsR was degraded *in vivo* upon heat shock and only in strains where *mcsB*, *mcsA*, *clpC* and *clpP* are present (Kirstein et al. 2005). These observations strongly suggested that McsB-P can act as an adaptor protein, targeting CtsR for degradation by ClpCP. This was the case which was subsequently proven by experiments demonstrating that McsB in its activated kinase state targeted CtsR for degradation by ClpCP. Comparable to the adaptor activity of MecA (Kirstein et al. 2006), McsB interacting mostly with the NTD and less with the Linker-domain of ClpC, supporting the oligomerization and activation of ClpC and thereby concurrently targeted CtsR to the active ClpCP complex. When McsB-P was not interacting with substrate protein, it was itself degraded, like MecA, by the ClpCP complex (Kirstein et al. 2007).



**Figure 4.** 'CtsR regulation by heat and oxidative stress'. In non-stressed cells, CtsR represses transcription of class III heat shock genes (*clpP* and *clpE* not shown) by binding to operator sites (three filled triangles). The kinase activity of MscB is inhibited by ClpC. Heat stress can be directly sensed by CtsR, resulting in its release from the DNA. This leads to derepression of class III heat shock genes. At the same time, the inhibitory interaction of ClpC with MscB can be disrupted by protein misfolding stress (e.g. by unfolded proteins targeted for degradation by ClpCP/MecA) resulting in MscB autophosphorylation in the presence of MscA. Phosphorylated MscB further contributes to the inhibition and release of CtsR from DNA and targets CtsR for degradation by ClpCP. MscA is oxidized and presumably proteolytically processed upon redox stress and dissociates from MscB. Monomeric free MscB, which is not phosphorylated under these conditions, inhibits and releases CtsR from DNA. Class III heat shock genes are de-repressed.

It should be noted, that the adaptor protein activity of MscB is regulated by phosphorylation of MscB (Kirstein et al. 2007), which is induced by stresses like heat (Kirstein et al. 2008) (Fig. 4). Likewise, the adaptor protein activity of MecA is also regulated, hence by a different anti-adaptor protein mechanism, induced by quorum-sensing pheromones (Turgay et al. 1998).

Protein arginine phosphorylation was first recognized in *B. subtilis* cells by the discovery that the protein kinase MscB (Kirstein et al. 2005) is actually phosphorylating specific arginines of the repressor protein CtsR (Fuhrmann et al. 2009).

In *B. subtilis*, the different proteins involved in protein arginine phosphorylation are MscB, the activator of MscB protein kinase activity MscA, the AAA+ protein ClpC that can inhibit MscB kinase activity (Kirstein et al. 2005; Elsholz et al. 2011a) and YwlE the phosphatase of arginine phosphorylated proteins (Kirstein et al. 2005; Elsholz et al. 2012; Fuhrmann et al. 2013). MscB displays a low autokinase activity, which is induced by MscA, resulting in a kinase-active autophosphorylated MscB, which also phosphorylates MscA and interacting substrate proteins such as CtsR (Kirstein et al. 2005). The first identified substrate of MscB kinase activity was the heat shock repressor CtsR, whose DNA-binding activity is inhibited by MscB and MscA and targeted by the activated MscB kinase for degradation to the ClpCP AAA+ protease complex (Kirstein et al. 2005, 2007; Fuhrmann et al. 2009) (Fig. 4).

The early experiments characterizing the protein kinase activity of this system suggested that MscB phosphorylates tyrosine, which was based on experiments (i) utilizing thin-layer chromatography to identify phosphorylated amino acids of an acid hydrolysate of *in vitro* phosphorylated MscA and MscB protein, where phosphorylated tyrosine was detected. (ii) YwlE, a member of the family of low-molecular-weight protein-tyrosine phosphatases (Musumeci et al. 2005; Xu, Xia and Jin 2006), was identified as the cognate phosphatase and (iii) certain variants of MscA or MscB where specific tyrosine residues replaced by phenylalanine abrogated the kinase activity of MscB (Kirstein et al. 2005).

In hindsight, it is very likely that the experimentally detected phospho-tyrosines were observed, because the transfer of a phosphate from the high-energy bond of an arginine-phosphate phosphoramidate to a relatively low-energy phosphoester in tyrosine is thermodynamically favored. The experimental conditions, in which *in vitro* phosphorylated MscB and MscA proteins were acid hydrolyzed overnight, could favor such a transfer (Kirstein et al. 2005). In support of this hypothesis, it was observed that in ClpC a tyrosine next to a phosphorylated arginine could become phosphorylated in the presence of MscB, but that this tyrosine phosphorylation was not observed in a ClpC variant, where the neighboring arginine was changed to Lys or alanine (Elsholz et al. 2012). In different

experiments, a transfer of phosphate from arginine to serine was directly observed (Schmidt, Ammerer and Mechtler 2013). Eventually, the unequivocal identification of the protein arginine phosphorylation of CtsR was established using more definite methods, including structural biology, mass-spectrometry and NMR (Fuhrmann et al. 2009).

Recently, the molecular mechanism of YwlE-mediated protein arginine phosphatase, and its relation to the original phospho-tyrosine phosphatase activity of this protein family from *B. subtilis*, was elucidated and characterized in molecular detail (Fuhrmann et al. 2013). The experiments presented in this study demonstrate that already very subtle amino acid changes in the active site of this phosphatase family (LMW-PTPs see Phosphotyrosine-protein phosphatases) could switch the phospho-tyrosine recognition of the LMW-PTPs to the more distinct phospho-arginine selectivity and recognition by the closely related YwlE phosphatase (Fuhrmann et al. 2013). This elegant study therefore clarifies on the molecular level how YwlE could evolve from a phospho-tyrosine into a phospho-arginine phosphatase, which is important to understand the role and possible function of this protein modification.

The kinase activity of McsB is linked to its ability to activate ClpC and target CtsR for degradation by ClpCP (Kirstein et al. 2007). Recently, it could be demonstrated both *in vitro* and *in vivo* that the phosphorylation of specific arginines of ClpC is necessary for the McsB-mediated activation, but not for the MecA-mediated activation of ClpC (Elsholz et al. 2012).

### Transcriptomics and Phosphoproteomics

Due to the relative instability of the protein arginine phosphorylation modification the *in vivo* identification of modified proteins is difficult and was only successful in *B. subtilis* cells lacking the YwlE phosphatase. In two different studies 87 or 134, proteins with 121 or 217 peptides phosphorylated at arginines could be identified by mass-spectrometry, but only a limited overlap between these putative modified proteins was observed (Elsholz et al. 2012; Schmidt et al. 2014).

Additional experiments including transcriptomics (Elsholz et al. 2012) and quantitative phosphoproteomics (Schmidt et al. 2014) suggested a regulatory impact of arginine phosphorylation in many pathways, other than the control of the CtsR regulon.

It should be mentioned that phosphoproteomic experiments in cells of a different Gram-positive organism, *S. aureus*, identified eight protein arginine phosphorylated peptides, even in the presence of the gene homologous to the YwlE phospho-arginine phosphatase (Bäsell et al. 2014). Four of these proteins were also identified as possible McsB substrates in *B. subtilis* [Tuf and GlnA by Schmidt et al. (2014) and RpoB and Glt by Elsholz et al. (2012)] (Bäsell et al. 2014). However, it should be noted that already a limited overlap of possible arginine-phosphorylated proteins in *B. subtilis* was observed (Elsholz et al. 2012; Schmidt et al. 2014) and that even the overlap of identified serine/threonine/tyrosine phosphorylation sites among different bacterial species is very low (Soufi et al. 2008).

More experiments to validate and explore the functional relevance or biological role for the observed protein arginine phosphorylation of the identified proteins have to be carried out in the future.

### Localization of McsB

Upon heat shock, autophosphorylated and activated McsB-P localizes, like ClpC, ClpX or ClpP, to foci formed by aggregated pro-

teins close to the poles (Kirstein et al. 2008). Such polar localization of McsB-P to protein aggregates might indicate that McsB could be an adaptor protein directly involved in recognizing unfolded or misfolded proteins, and targeting ClpC or ClpCP to intracellular protein aggregates in order to facilitate the disassembly and disaggregation of these intracellular structures formed by protein aggregates. This possible role of McsB is supported by the recent observation that a cryptic inactive enzyme GudB<sup>CR</sup> fused to GFP, which is instable and prone to misfolding (Gunka et al. 2012), strongly accumulates in foci at poles in cells missing McsB (Stannek et al. 2014), similar to foci formed by aggregated proteins (Runde et al. 2014).

A strikingly strong polar localization of the ComGA protein, which is part of the specific DNA receptor and transport complexes active during competence in *B. subtilis*, was observed in a *mcsB* mutant *B. subtilis* strain expressing a ComGA-GFP fusion protein. Further experiments demonstrated that McsB, McsA, ClpC and ClpE are necessary to disassemble these DNA receptor complexes during escape from the competence state (Hahn et al. 2009). The observed McsB-mediated disassembly of the competence DNA receptors (Hahn et al. 2009) would resemble a more general activity of disassembling protein aggregates, which is corroborated by the observation that ComGA was identified as a possible McsB kinase substrate (Elsholz et al. 2012).

### Different regulatory layers of stress sensing

For *B. subtilis* a number of different mechanisms to sense oxidative or heat stress, and transmit these signals to control the McsB and CtsR activity have been suggested (Kirstein et al. 2007; Fuhrmann et al. 2009; Elsholz et al. 2010, 2011a,b) and will be discussed in the following section. To understand this complex regulatory system one has to consider that inhibition of CtsR results in increased transcription and translation of McsA, McsB and ClpC, which all mutually modulate their own activity and that of CtsR. The latter activity is important to prevent the immediate shut down of this stress response pathway by newly synthesized CtsR repressor, but it also allows an appropriate control of the scope and duration of the stress response (Fig. 4).

CtsR, McsA, McsB and ClpC activities are regulated by different PTM mechanisms, including specific protein-protein interactions, phosphorylation and proteolysis. The interaction of McsB, CtsR, McsA and ClpC can have different and even opposite outcomes, depending on the phosphorylation state and activity of McsB itself but also on different activity states of McsA, ClpC and even CtsR.

McsB, even when not active as a kinase, inhibits the repressor activity of CtsR, and McsA activated McsB-P enhances *in vitro* the inhibition of CtsR (Kirstein et al. 2005). Most likely because CtsR is phosphorylated at arginine residues which are important for its interaction with DNA (Fuhrmann et al. 2009).

The interaction of McsB with ClpC also appears to have two different modes depending on the two possible activity states of McsB and ClpC. Monomeric ClpC inhibits the kinase activity of McsB (Kirstein et al. 2005; Elsholz et al. 2011a). Yet, kinase-active McsB-P acts as an adaptor protein for ClpC that facilitates the activation and oligomerization of ClpC, which is a requirement for the formation of the ClpCP AAA+ protease complex. During this process, McsB-P binds to CtsR so that it is unfolded by ClpC and subsequently degraded by the associated ClpP. This further



enhances the inhibition of CtsR by McsB in the presence of ClpC (Kirstein et al. 2005). However, in the absence of CtsR or other substrate proteins, McsB-P is itself degraded by ClpCP, which allows to control and limit the cellular level of kinase active McsB (Kirstein et al. 2007) (Fig. 4).

Based on these results, it was suggested that ClpC inhibited the switch between McsB to activated McsB-P that targets CtsR for degradation (Kirstein et al. 2007; Elsholz et al. 2011a). This switch-like mechanism could enable a heat sensing mechanism, where the inhibitory ClpC-McsB complex is dissipated through the presence of heat acting as a trigger to induce unfolded or aggregated proteins. The breakup of this inhibitory complex would enable the transformation of McsB by McsA into activated McsB-P, which subsequently oligomerizes and activates ClpC. This model of a heat sensing mechanism was based mostly on experiments with purified proteins (Kirstein et al. 2005, 2007), but was later confirmed by *in vivo* experiments (Elsholz et al. 2011a) (Fig. 4).

In addition to this signaling event, yet another layer of regulation and stress sensing was uncovered. Based on the CtsR structure (Fuhrmann et al. 2009) a glycine rich loop of CtsR was identified, which can sense temperature and modulate DNA-binding activity (Elsholz et al. 2010). Subsequently it was demonstrated *in vitro* and *in vivo* that CtsR itself can become inactivated by heat and thereby can sense directly increased temperature. This heat inactivated CtsR does not need not be inhibited by McsB anymore, though it is still targeted by McsB-P for ClpCP degradation (Elsholz et al. 2010) (Fig. 4).

In addition, Elsholz and colleagues demonstrated that, when *B. subtilis* cells were exposed to various oxidative stresses, CtsR inactivation depended on the presence of McsB but not its kinase activity. It could be demonstrated that under these conditions stress sensing was mediated by oxidation of McsA, which resulted in a release of McsA from McsB. The authors postulated that McsA, when interacting with McsB, can also be an inhibitor for a kinase-independent CtsR repressing activity of McsB, which is relieved upon oxidation of McsA (Elsholz et al. 2011b). In this model, McsA interaction with McsB has opposing effects on the two different kinase-inactive and kinase-active McsB states (Elsholz et al. 2011b) (Fig. 4).

The recurring theme of this complex regulatory system appears to be the different interactions of McsB and kinase-active McsB-P with its respective interacting proteins that can also switch between two states. For example, when McsA activates McsB it becomes phosphorylated, and ClpC is also phosphorylated during activation by McsB-P. Therefore, phosphorylation by the McsB kinase might enable these different states and activities of ClpC or McsA.

YwlE, as the cognate protein arginine phosphatase, dephosphorylates McsB-P, and is therefore involved in the McsB-mediated regulation (Elsholz et al. 2012). YwlE very likely also plays a role in stress sensing, since McsB is switched to a kinase active state in the absence of heat stress and McsB-P is also destabilized and prematurely degraded in a *ywlE* mutant strain (Kirstein et al. 2008; Elsholz et al. 2010).

It is interesting to note that an *ywlE clpC* mutant strain displays a severe growth defect that depends on the kinase activity of McsB, which suggests that uninhibited McsB kinase activity can be detrimental for growth and survival of *B. subtilis*. One reason could be an unrestricted protein arginine phosphorylation activity of McsB, which would interfere with the activity of many proteins normally not phosphorylated by McsB (Elsholz et al. 2011a).

## Evolution of protein arginine phosphorylation

McsB contains a guanidine kinase domain, homologous to phosphagen kinase domains of mostly eukaryotic creatine or arginine kinases, which can catalyze the reversible phosphotransfer from ATP to arginine to allow buffering the cellular ATP levels (Ellington 2001; Kirstein et al. 2005; Fuhrmann et al. 2009). It was recently observed that a little number of bacterial species like *Myxococcus* mostly from proteobacteria encode such phosphagen kinases. The buffering of the cellular ATP levels by arginine kinases might be beneficial for cellular stress responses also in bacterial cells, as observed for *Myxococcus* cells lacking the gene for arginine kinase, which also display developmental defects (Andrews et al. 2008; Bragg et al. 2012; Suzuki et al. 2013). The phosphagen kinase domain of McsB homologs appears to be closer related to these recently identified bacterial arginine kinases than to the eukaryotic homologs, but lacks the arginine recognition domain essential for the arginine kinase activity. However, the McsB homologs are detected mostly in firmicutes with a possible occurrence in organisms of the PVC phylum (Bragg et al. 2012; Suzuki et al. 2013).

When examining the cooccurrence of genome encoded proteins with McsB using the String website (Szklarczyk et al. 2015), it becomes apparent that genomes encoding a McsB homolog almost always also encode homologs of McsA, YwlE and ClpC especially in firmicutes. Since these proteins control the kinase activity of McsB, their presence would possibly allow and enable the establishment of cellular protein arginine phosphorylation during evolution. This would also be consistent with the observation that unrestricted protein arginine phosphorylation by McsB can be detrimental for the cellular function.

In this respect, YwlE seems to be very efficient since with our current methods the relative transient phospho-arginine protein modification could only be detected in the presence of a phosphatase inhibitor, or in cells lacking the gene for this specific phosphatase in *B. subtilis* cells (Elsholz et al. 2012; Schmidt et al. 2014). We already mentioned that YwlE is a member of the highly conserved phospho-tyrosine phosphatase family, which developed into a phospho-arginine phosphatase (Mijakovic et al. 2005; Musumeci et al. 2005; Fuhrmann et al. 2013). This is an interesting connection between the bacterial tyrosine and arginine phosphorylation system via a common type of phosphatase and could suggest that the *B. subtilis* protein arginine phosphorylation system might have somehow appropriated the activity of the ubiquitous LMW-PTP protein family during evolution.

However, a more in depth analysis of the distribution of the YwlE phospho-arginine phosphatase homologs (Fuhrmann et al. 2013) in all currently available genomes (Johnson et al. 2008) revealed that although all the organisms encoding the YwlE homolog also encoded an McsB homolog, only less than a quarter of the organisms encoding McsB also encoded this specific YwlE homolog. This suggests that different strategies and proteins to control the protein arginine phosphorylation activity of McsB might have been utilized during evolution, possibly including different phospho-arginine phosphatases or McsB activity controlling proteins such as McsA or ClpC.

## Protein arginine phosphorylation functions as part of the protein quality control system

One could consider the described protein arginine phosphorylation system as a recently adapted protein modification

system on the brink of becoming also a regulatory system. Because of the transient nature of this modification, this system might mostly function for fast responses and transient protein activity modulations.

Evidently, the transient arginine phosphorylation appears to allow functional conformational switches e.g. in McsB, ClpC and possibly McsA. But the concurrent protein arginine phosphorylation of protein substrates by the adaptor protein McsB, which is also kinase active, might not be essential for the functions of many of these modified proteins. The concomitant protein modification could nevertheless help to inactivate, modify or mark these proteins or protein complexes, which are to be disassembled or might already be misfolded, damaged or aggregated.

The dual involvement of McsB and ClpC in regulation and protein quality control might also be reflected in the identification of specific arginine phosphorylation sites controlling the activation of ClpC (Elsholz et al. 2012), and the additional identification of many possible ClpC arginine phosphorylation sites, which should hold this chaperone inactive (Schmidt et al. 2014).

However, the observed pleiotropic regulatory impact of protein arginine phosphorylation beyond the CtsR regulon (Elsholz et al. 2012; Schmidt et al. 2014) suggests that new additional regulatory substrates of McsB are bound to be discovered and characterized in future experiments.

## DUAL ROLES, INTERCONNECTION AND INTERPLAY BETWEEN BACTERIAL PHOSPHORYLATION SYSTEMS

### Possible dual functions of bacterial kinases and phosphatases

It is interesting to note that some of the bacterial kinase and phosphatase display dual activities and roles as discussed for the Kinase/phosphatases HprK/P and IDHK/P and McsB.

The protein kinase McsB is also an adaptor protein for regulatory and general proteolysis thereby directly connecting two different signal transduction systems, especially important for stress responses. Interestingly, both systems can act in a fast and reversible manner and both systems can be complementary due to their different mechanisms. The different roles of phosphorylation and regulated proteolysis mediated by McsB are currently sorted out. However, other systems that connect two-component systems and regulatory proteolysis are known. For example, the activity of the global regulator CtrA of *C. crescentus* is controlled both by phosphorylation and regulatory proteolysis (Domian, Quon and Shapiro 1997; Jenal and Fuchs 1998; Abel et al. 2011; Joshi et al. 2015).

Interestingly, a dual function of BY-kinases was also discussed, because before their characterization as tyrosine-kinases, some of these enzymes were originally thought to function as ATPase energizing the capsule assembly machinery (Whitfield and Roberts 1999). This possible function was considered much less likely, since the tyrosine-kinase activity of BY-kinases has been demonstrated. Nevertheless, it was observed in a study about the BY-kinase Wzc from *E. coli* that phosphoablative mutations in the tyrosine cluster do not induce the same defects as a mutation in the Walker A motif (Obadia et al. 2007; Soulat et al. 2007). Therefore, one cannot exclude that BY-kinases could also function as authentic ATPases besides their kinase activity. This would imply that their kinase and ATPase functions would be tightly coordinated and regulated to optimize the organization and the dynamics of the capsule assembly

machinery. Along the same line, the extracellular domain of BY-kinases shares similarities with Wzz proteins that act as chain-length regulator for lipid A-core-linked polysaccharides (Dodgson, Amor and Whitfield 1996). One can thus not exclude that beside its role in ligand binding and kinase activation (Elsholz, Wacker and Losick 2014); the extracellular domain of BY-kinases could also influence the activity of the polymerase of the capsule assembly machinery through direct protein-protein interactions.

A dual function could also be discussed for BY-kinase cognate LMW-PTPs, which were proposed to dephosphorylate undecaprenol phosphate, the lipid carrier on which capsular polysaccharides are assembled (Bugert and Geider 1997). However, this hypothesis has not been further investigated since LMW-PTPs were shown to dephosphorylate BY-kinases. Knowing that this lipid carrier molecule is also required for peptidoglycan as well as cell wall teichoic acid synthesis (Bouhss et al. 2008), one can not exclude that LMW-PTPs might be in addition associated with the regulation of the amount and availability of the lipid carrier for capsule, peptidoglycan and teichoic acid synthesis.

### Cross-talk of bacterial protein modification systems

The mechanism and role of bacterial serine/threonine and tyrosine phosphorylation appear to be very different. The specific role of unusual serine/threonine and tyrosine phosphorylation in the control of enzymatic activity for metabolism or biofilm formation was discussed and described. How the bacterial tyrosine kinases such as PtkA (EpsB) are integrated in signal transduction networks is only beginning to be resolved.

However, additional layers of regulation between the different phosphorylation systems, such as cross-phosphorylation of protein kinases, are increasingly observed in many bacteria. For example, in *M. tuberculosis* cross-phosphorylation was reported among Hanks-type serine/threonine kinases (Baer et al. 2014), and it was also shown that Hanks-type serine/threonine kinases can be phosphorylated by protein-tyrosine kinases (Kusebauch et al. 2014). Importantly, Kusebauch and colleagues demonstrated that tyrosine phosphorylation of *M. tuberculosis* Hanks-type kinases regulates their activity *in vitro* and *in vivo* (Kusebauch et al. 2014).

In *B. subtilis*, cross-phosphorylation by kinases, involving BY-kinases, Hanks-type kinases (eSTK), two-component-like serine/threonine kinases, HprK/P and response regulators was observed for different signal transduction pathways (Shi et al. 2014b; Libby, Goss and Dworkin 2015; Mijakovic and Deutscher 2015). Many of these cross-phosphorylation events may have a distinct regulatory role. Nevertheless, the exact interplay and functional impact of cross-phosphorylation of these different pathways have to be investigated in more detail (Gerwig and Stülke 2014).

Besides the strong homology between phospho-arginine and phospho-tyrosine phosphatases (Fuhrmann et al. 2013) of these otherwise very different phosphorylation systems, it was observed that the high-energy phosphate of phosphorylated arginines could be directly transferred to form a lower-energy phosphoester bond on neighboring serines or tyrosines (Elsholz et al. 2012; Schmidt, Ammerer and Mechtler 2013). If this cross-phosphorylation really takes place *in vivo*, then this process might be considered as a kind of chemical cross-talk between arginine and tyrosine phosphorylation. But the possible biological significance of such a cross-talk has yet to be demonstrated.

In the complex landscape of PTMs, cross-talk among different types of kinases is just a tip of the iceberg. It is a

well-established fact that different PTMs on the same polypeptide can affect each other (Beltrao et al. 2012, 2013; Minguez et al. 2012; van Noort et al. 2012). A well-known example is the interplay among methylation, phosphorylation, acetylation and other modifications of the histone proteins (Izzo and Schneider 2010). In essence, the occupancy of one modified site can affect the accessibility of another site on the same protein (Beltrao et al. 2013). Interestingly, it was recently observed that post-translationally attached protein modifications such as ubiquitin can themselves be modified by phosphorylation (Koyano et al. 2014; Herhaus and Dikic 2015; Wauer et al. 2015).

In bacteria, the interplay of different PTM's has been less explored. First evidence of direct influence of protein acetylation on protein phosphorylation, and vice versa, came from a study in *Mycoplasma pneumoniae*, a bacterium with a minimal genome. Here, deletion of protein kinases affected the composition of the acetylome, whereas deletion of *N*-acetyltransferases perturbed the composition of the phosphoproteome (van Noort et al. 2012). Additional evidence from global studies of acetylation and phosphorylation in other bacteria, suggests that this may be a widespread phenomenon (Soufi et al. 2012).

## CHALLENGES AND PROGRESS

Gel-free site-specific phosphoproteomics approaches provide a reliable platform for global detection of protein phosphorylation on e.g. tyrosine or even arginine in bacteria (Macek et al. 2007, 2008; Elsholz et al. 2012; Schmidt et al. 2014). However, most of protein phosphorylation sites detected by phosphoproteomics have not yet been analyzed and shown to have an effect on protein function. Nevertheless, recent experiments performed with *S. pneumoniae* on the cell division protein DivIVA and MapZ and LytA illustrate that information derived from global phosphoproteomic approaches represents a promising source for understanding the impact of phosphorylation on bacterial protein function (Fleurie et al. 2012, 2014; Standish, Whittall and Morona 2014).

For the phosphoproteome of arginine phosphorylation, this global approach has to be taken with a grain of salt. Due to the transient chemical nature of protein arginine phosphorylation such a global phosphoproteome analysis could only be achieved in *B. subtilis* strains lacking or inhibiting the phosphatase (Elsholz et al. 2012; Schmidt et al. 2014). Unfortunately, the lack of this phosphatase makes it also difficult to assess the nature and the relevance of the identified protein modification and its possible regulatory or physiological *in vivo* role. For example, for at least two identified modifications by protein arginine phosphorylation in ScoC or FlgN, no significant *in vivo* role could be confirmed in *B. subtilis* (Cairns et al. 2014; Belitsky et al. 2015).

Furthermore, McsB kinase activity is intricately connected to its function as adaptor protein for the AAA+ protein ClpC (Kirstein et al. 2007). The kinase-active McsB might therefore not only be involved in the regulation of heat stress response but could, together with ClpC, also participate in the general disassembly or disaggregation of protein aggregates (Kirstein et al. 2008; Hahn et al. 2009). This could suggest that a number of the identified proteins phosphorylated at arginines were identified because they are misfolded or aggregated.

New efforts to improve the detection of peptides carrying phosphorylated arginines by mass spectrometry (Schmidt, Ammerer and Mechtler 2013) or the development of protein

tools to specifically recognize arginine-phosphorylated proteins are highly warranted and required to better investigate this unusual protein modification (Fuhrmann et al. 2013; Trentini et al. 2014). An important step in this direction has been taken, since the generation of a functioning anti-phosphoarginine-specific antibody using synthetic phosphonate amidines as templates was recently reported (Fuhrmann, Subramanian and Thompson 2015).

It is interesting to note that removal of a single phosphatase allowed a phosphoproteome analysis of phospho-arginine modified proteins, which is most probably not feasible for the more complex two-component systems comprising the other chemically more transient protein modifications: histidine and aspartate phosphorylation. Here, a variety of different specific mechanisms and enzymes including the histidine kinases themselves and not one phosphatase are controlling the flow of phosphates (Laub and Goulian 2007; Mitrophanov and Groisman 2008; Gao and Stock 2009; Salazar and Laub 2015).

With the current detection techniques, low abundance of phosphopeptides remains another important challenge. A very broad range of peptide abundance in the proteome can easily mask some phosphopeptides. Therefore, optimizing sample preparation and enrichment techniques are still an absolute requirement, irrespectively whether the gel-free or gel-based techniques are used (Bäsell et al. 2014; Lin, Sugiyama and Ishihama 2015; Zheng et al. 2015). Another relevant challenge for phosphoproteomics is the requirement to go beyond a single bacterial species and examine interactions between different bacteria or bacterial pathogens and the human host. In those cases the detection challenges are multiplied by sample complexity (Kluge et al. 2012).

Recently, interactomics has been demonstrated to be a useful tool for charting the interaction network comprising bacterial protein kinases and their substrates (Shi et al. 2014c). It should be pointed out that a network derived from interactomics differs significantly from the one based on proteomics. Since results of both have been shown to be physiologically relevant, the inevitable conclusion is that these two approaches are complementary, and should be used in tandem.

It should be pointed out that many phosphorylation sites appearing with low stoichiometry and on abundant proteins may be of little physiological relevance (Levy, Michnick and Landry 2012). However, low stoichiometry is not necessarily an indicator of physiological irrelevance, since activating effects of protein phosphorylation can be quite dramatic, even with low occupancy of phosphorylation sites. An example for this is the *B. subtilis* UDP-glucose dehydrogenase Ugd, which was shown to be phosphorylated to a level of 5% (Mijakovic et al. 2003), but the consequence of this phosphorylation was a 6.5-fold increase in  $k_{cat}$  (Petranovic et al. 2009).

Different phosphoproteome studies revealed that the overlap of Ser/Thr/Tyr phosphorylation substrates can be very low among different even closely related species. These observed changes and differences in phosphorylation patterns suggest that reversible protein phosphorylation could be a very amenable and useful epigenetic mechanism for adaptation of closely related organisms to specific niches and environments. While the conservation of actual phosphorylation sites is low, several classes of proteins are typically frequently phosphorylated in bacteria. For example, glycolytic enzymes (Soufi et al. 2008), enzymes involved in carbon metabolism, ribosomal proteins and other proteins involved in translation are typically phosphorylated in all reported bacterial phosphoproteomes (Mijakovic and Macek 2012). This could suggest that reversible



phosphorylation regulates or fine-tunes some of the key processes for cell survival and adaptation.

Consistent with this adaptation hypothesis, phosphoproteome content can change dramatically in different growth conditions (Prisic et al. 2010). Even in the same defined medium, sampling the bacterial culture at different time points along the growth curve leads to drastically different phosphoproteomes (Ravikumar et al. 2014). Being a true signal transduction device, reversible protein phosphorylation is a dynamic event. Gaining a complete overview of its dynamics will require performing time-resolved quantitative phosphoproteome analysis with many time points (Francavilla et al. 2014), covering relevant physiological conditions for every bacterial species. A more recent example for such a time-resolved approach is the analysis of the dynamic phosphoproteome of germinating *B. subtilis* spore cells, which suggests a functional connection of protein phosphorylation and the modulation of this cellular development program (Rosenberg et al. 2015).

This is not a trivial challenge. Single-cell quantitative phosphoproteomics is still not technically possible due to the amount of required protein. Therefore, sample heterogeneity due to bistability and heterologous cell populations remains a major challenge (Maamar and Dubnau 2005; Smits et al. 2005; Dubnau and Losick 2006; Smits, Kuipers and Veening 2006). Synchronization techniques are an interesting approach to address some of these problems.

The final verdict in each individual case must come from in-depth physiological characterization of phosphorylated proteins, and for doing this there is currently no reliable high-throughput alternative to classical molecular biology and biochemistry studies.

## CONCLUSIONS

The nature of the recently discovered bacterial protein networks utilizing tyrosine, serine or arginine phosphorylation, we described in this review, display fundamental differences not only due to the different sometimes dual roles, mechanisms or chemical differences of the phosphorylated amino acid residues. Nevertheless, recent research increasingly suggests that these different cellular phosphorylation systems appear to be able to communicate possibly allowing additional regulatory layers.

It should be kept in mind that these different phosphorylation systems with their distinct and overlapping roles, and mechanisms are not only allowing fast stress responses but could also be considered as versatile epigenetic response systems, which could be important for the first adaptation of an organism to different niches and specific environments.

Maybe therefore microorganisms with their wide variety of life styles and environments appear to be a great resource to identify and study different and distinct protein modification systems, like the new classes of bacterial tyrosine and unusual serine kinases or the recently discovered protein modification by arginine phosphorylation we described and discussed.

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